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THE ANALYSIS OF FLAVONOIDS AS POSSIBLE OESTROGENIC COMPOUNDS IN THE DIETS OF QUAIL

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The Analysis of Flavonoids as Possible Oestrogenic Compounds In the Diets of Quail

Final Report
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RADIAN
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FINAL REPORT

MAY 1982

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ABSTRACT

This report presents the results of a study to investigate the role of flavonoids as possible oestrogenic compounds in the food plants of quail from two disjunct sites in west Texas. Quail show dramatic differences in reproductive fecundity from year to year. Dry years lead to reduced fecundity. Samples of quail food plants and moisture data were taken over the period from mid-January to mid-June, 1981. The food plants were analyzed to determine if isoflavonoids, known to be oestrogenic in mammalian metabolism, were present and to determine if correlations existed between flavonoid concentrations and moisture stress in the environments of the two sites. Samples were analyzed by paper chromatography and relative flavonoid concentrations determined by thin-layer chromatography/in-situ absorbance densitometry. In addition, the major flavonoids of one species, Astragalus nuttallianus, were analyzed by ultraviolet spectrophotometry. From the study it is concluded that it is highly unlikely that isoflavonoids comprise a major element of the flavonoid loading in the quails' diet. In addition, statistical analyses of the data show that in general, flavonoid concentrations showed negative correlations with reduced available soil moisture and that little difference existed in flavonoid concentrations at the two sites for the test period.

1.0 INTRODUCTION

This research was undertaken in support of a program for the United States Department of Agriculture (USDA) Forest Service in cooperation with the Rocky Mountain Forest Range Experiment Station and the Great Plains Wildlife Research Laboratory in Lubbock, Texas. The purpose of the study was to investigate flavonoids as potential reproductive inhibitors in quail food plants. Dramatic differences in quail reproduction occur from year to year, apparently in response to environmental factors. Reproductive fecundity decreases in dry years (Ref. 1).

It is suspected that flavonoids in plants may act as oestrogenic compounds in the quails' diet. Phytoestrogenic compounds have been shown to inhibit fertility in domestic sheep and rodents. The active compounds have been shown to be isoflavones found in Trifolium subterraneum and T. pratense (subterranean and red clovers) (Ref. 2, 3 and 4). The principle active compounds were formononetin, genistein, and biochanin A. In addition, oestrogenic activity has been detected in T. repens (ladino clover) and Medicago sativa (alfalfa) (Ref. 5). In this case, the active components were shown to be coumestrol and other coumestans. While the coumestans are more active than other isoflavones, they have been detected at much lower concentrations in forage plants.

In addition to the isoflavones, kaempferol 3-rhamnosylgalactoside-7-rhamnoside and quercetin have been shown to have weak oestrogenic activity (Ref. 6). Hesperetin and morin have been shown to block the effects of progesterone in laboratory studies using isolated rabbit uterus (Ref. 7).

This research was undertaken to attempt to evaluate the potential role of flavonoids as oestrogens in the quails' diet. Samples of quail food plants were taken from two disjunct sites in west Texas to determine first if isoflavonoids were present and second, if total flavonoid content showed correlations to moisture stress in the environment.

2.0 RESULTS, CONCLUSIONS AND RECOMMENDATIONS

This section presents a brief project summary. In addition, qualitative and quantitative results are presented along with the conclusions and recommendations based on these results.

2.1 Project Summary

The United States Department of Agriculture (USDA) Forest Service Rocky Mountain Forest Range Experiment Station is conducting a study to determine factors which may effect the reproductive fecundity of quail. This fecundity varies dramatically with relatively wet and dry years. Dry years produce reduced fecundity (Ref. 1).

This project was undertaken to determine if the flavonoid contents in the quail food plants vary with moisture stress in the environment and, therefore, are correlated to quail reproductive fecundity. USDA personnel sampled quail to determine major food plants in their diet over the period of mid-January to mid-June in 1981 at two disjunct sites in west Texas. The Andrews site is located approximately 8 miles northwest of Andrews, Texas, and the Bailey site about 15 miles southwest of Muleshoe, Texas. The food plants were then collected for flavonoid analysis. Sampling is discussed in detail in Section 3.0.

In addition to the collection of food plants, moisture stress in the environment at the two sites was determined by USDA. Rainfall accumulations and subsequent soil moisture were determined by measuring rainfall

and moisture readings at five stations scattered about each of the collection sites. Soil moisture measurements were made at 4, 16 and 28 cm.

The purpose of this study was to provide analytical support for the USDA. The goals of the analytical program were:

- o to determine if isoflavonoids are present in the quails' diet,
- o to determine if correlations exist between moisture stress and intraspecific flavonoid content within and between the two sites, and
- o to determine if correlations exist between moisture stress and total flavonoid intake within and between the two sites.

In order to examine the possibility of these phoenomena a total of 51 plant samples from these 2 sites collected over a 6-month period were taken for subsequent analyses. These samples represented 15 genera in 11 families.

The analytical approach, discussed in detail in Section 4.0, used the following techniques:

- o initial extract characterization using paper chromatography,
- o scanning ultraviolet spectrophotometric characterization of flavonoids from one plant species, and
- o thin-layer chromatography with in-situ quantitation of flavonoids by absorbance densitometry.

The results of this effort are discussed in the following sections.

2.2 Analytical Results and Conclusions

Results of the initial extract characterizations by paper chromatography, characterizations of the flavonoids of Astragalus nuttallianus by scanning ultraviolet spectrophotometry and quantitation of the flavonoids in the plant samples by thin-layer chromatography/absorption densitometry are discussed below.

2.2.1 Paper Chromatography

The crude methanol:water extracts of the plant samples were subjected to paper chromatography to provide an initial characterization of the flavonoid constituents of the samples and to determine if additional fractionation would be required for quantitative analysis. Paper chromatography was performed using the standard methods of Mabry, Markham and Thomas (Ref. 8). Chromatograms were viewed prior to and after fuming with ammonia using ultraviolet (UV) light at a wavelength of 370 nm.

Initial qualitative data may be obtained using this technique. Degree of glycosylation and general structural type may be assessed by examining Rf values for the compounds and their colors and color changes

when viewed with UV light before and after fuming with ammonia. The general distribution of flavonoid types expected in paper chromatographic analysis is shown in Figure 2-1 taken from Reference 8.

The paper chromatograms were examined by the author and Dr. T. J. Mabry of the Department of Botany at The University of Texas at Austin.

The following conclusions were drawn from the chromatographic analysis:

- The presence of isoflavonoids was unlikely except in the case of Astragalus nuttallianus.
- With the above exception, the chromatograms indicated typical flavonoid patterns including flavone, flavonol glycosides and, in some cases, flavone aglycones.
- Paper chromatographic resolution indicated that further extract fractionation would not be necessary for subsequent analysis by thin-layer chromatography.

The unlikelihood of the occurrence of isoflavonoids in other species represented in the quails' diet is further supported by a review of the literature (Ref. 9). Table 2-1 lists the families and genera represented in the quails' diet. Note that of 15 genera represented, only 1, Astragalus occurs in the Leguminosae. Table 2-2 (compiled from Ref. 9), reports the number of isoflavonoid structures which have been reported, the number of occurrences and the plant sources. Of a total of 771 reported

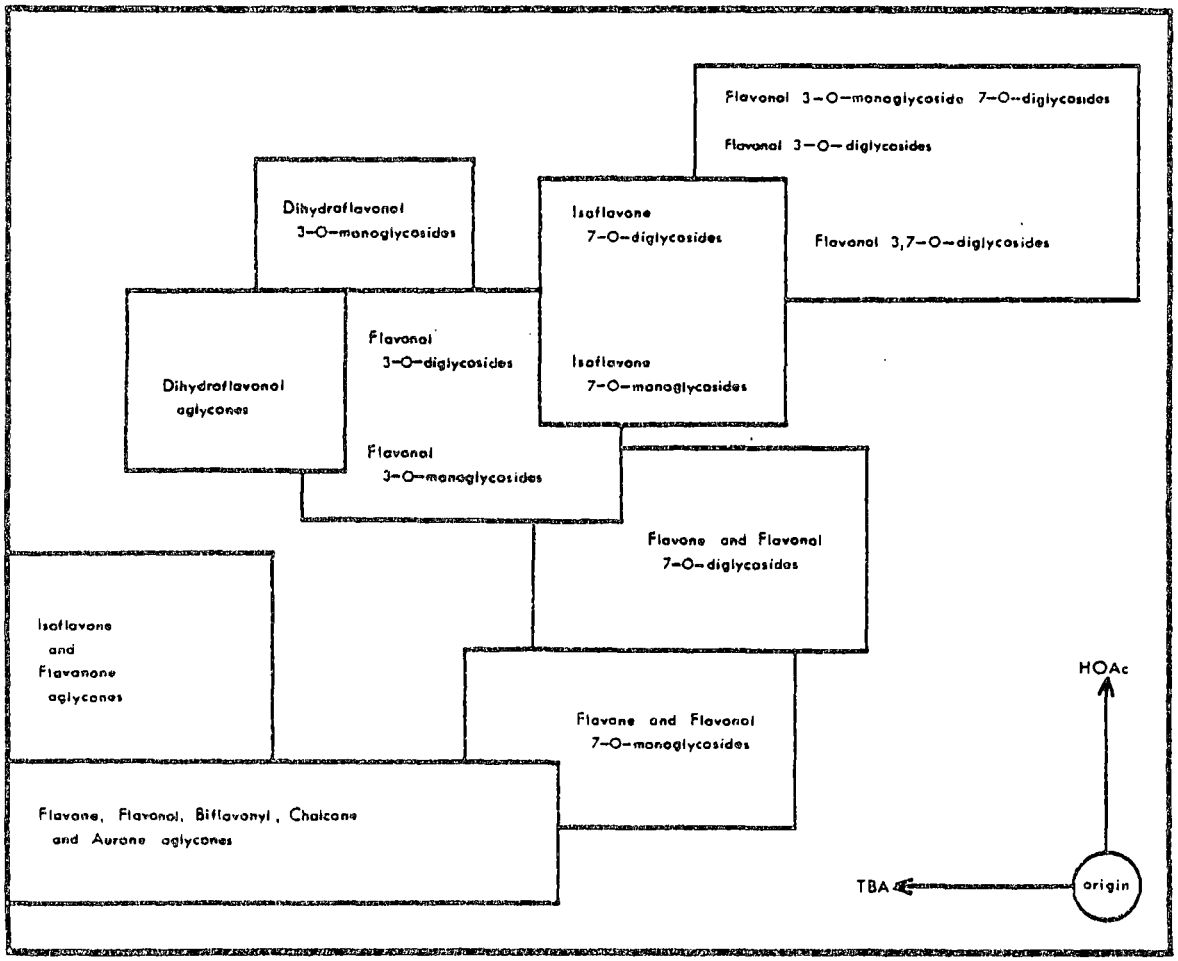


Figure 2-1. The Distribution of Flavonoids on a TBA/HOAc, Two-Dimensional Paper Chromatogram

Table 2-1. Genera and Families Represented in the Diets of West Texas Quail

Family	Genus
Boraginaceae	Lithospermun Cryptantha
Chenopodiaceae	Kochia
Compositae	Hymenopappus Machaeranthera Ambrosia
Cruciferae	Descurainia Lepidium Lithospermum
Leguminosae	Astragalus
Liliaceae	Allium
Malvaceae	Sphaeralcea
Onagraceae	Oenothera
Polygonaceae	Eriogonum
Solanaceae	Physalis

Table 2-2. Reported Occurance of the Classes of Isoflavonoids

Isoflavonoid Type	Number of Compounds Reported	Total Number of Occurrences	Total Number of Occurrences Exclusive of Leguminosae	Sources Exclusive of Leguminosae
Isoflavones	183	366	62	Chenopodiaceae Cupressaceae Iridaceae Myristicaceae Rosaceae Aspergillus Streptomyces
Isoflavanones	30	34	0	None
Rotenoids	40	76	3	Stemonaceae
Pterocarpans	85	169	2	Myristicaceae
Isoflavans	32	87	0	None
Isofavanquinones	6	8	0	None
Isoflav-3-enes	6	6	0	None
Coumestans	4	16	0	None
2-Arylbenzofurans	7	9	0	None
Totals	397	771	67	

Compiled from Reference 9.

occurrences of 397 different isoflavonoids, only 67 occurrences have been reported outside of the Leguminosae. Of these 67, only 1 occurrence was reported in any other family represented in the quails' diet. This was betavulgarin reported from Beta vulgaris in the Chenopodiaceae. The family Chenopodiaceae is represented by only one species, Kochia scoparium, in the quails' diet. Both the paper and thin-layer chromatograms of Kochia indicated the presence of only one or two highly glycosylated compounds which eluted where flavonol triglycosides would be expected. Therefore, from both chromatographic results and literature reports it was concluded that it was highly unlikely that any plant in the quails' diet except Astragalus nuttallianus contained isoflavonoids.

2.2.2 Scanning Ultraviolet Spectrophotometry

However, both the common occurrence of isoflavonoids in the Leguminosae and the unusual distribution of flavonoids on the paper chromatogram for Astragalus (shown in Figure 2-2) indicated the need for further investigation of the flavonoids of this species. Because the paper and thin-layer chromatograms for all Astragalus collections indicated that the same compounds were present in all samples, it was concluded that data needs could be met by examining one representative sample. The sample used was collected at the Andrews site on April 22, 1981.

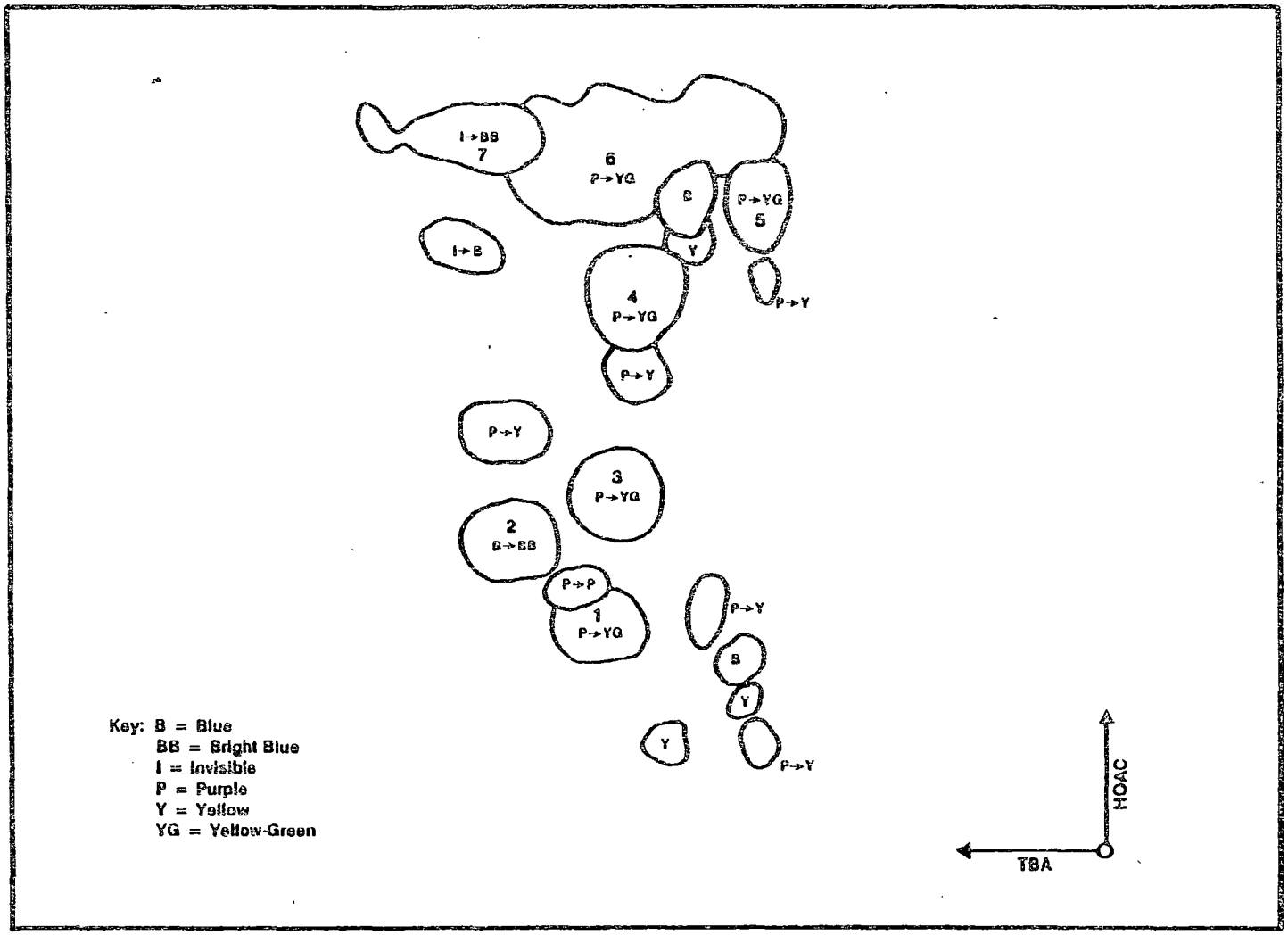


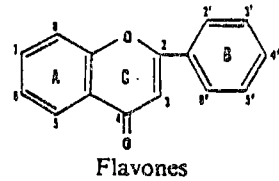
Figure 2-2. Two-Dimensional Paper Chromatogram of Astragalus nuttallianus

Scanning ultraviolet spectrophotometry (UVS) was chosen as the analytical method for further investigation of the Astragalus sample. The limited sample size available for analysis made UVS the only practical technique available since relatively small quantities of compounds may be quantitated with this technique. In addition, while UVS is limited by some ambiguity in confirming the presence of isoflavonoids, there is little ambiquity in confirming their absence.

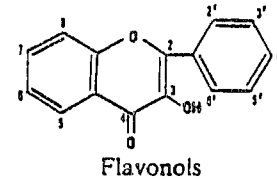
The more common classes of flavonoids, the flavones and flavonols, and two of the less common types, aurones and chalcones, shown in Figure 2-3, exhibit two major absorption bands, one in the range of 240 - 280 nm and one typically in the range of 300 - 380 nm. These bands are referred to as Band II and Band I respectively. However, the isoflavones exhibit a Band II absorbance only. Unfortunately this is also true of two other less common classes of flavonoids, the flavanones and dihydroflavonols. Therefore, there is some ambiquity in confirming the presence of isoflavonoids.

The spots which were present at sufficient concentrations for isolation by paper chromotography and subsequent UVS analysis are numbered one through seven in Figure 2-2. As the figure illustrates, five of the spots were purple while one was blue and one was invisible turning to blue with ammonia. A blue spot indicates typically a nonflavonoid phenolic compound or less frequently, a flavonoid lacking a free 5-OH group.

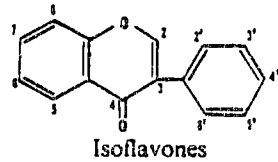
The UVS data for these spots are given in Table 2-3. The structural types suggested from the UVS data are given in Table 2-4. From these



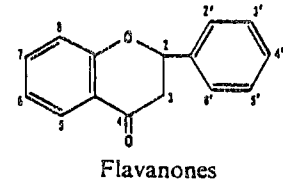
Flavones



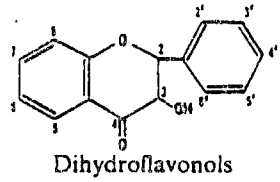
Flavonols



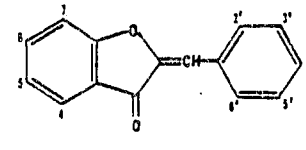
Isoflavones



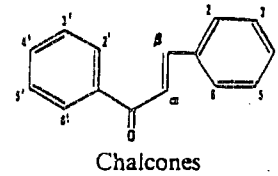
Flavanones



Dihydroflavonols



Aurones



Chalcones

Figure 2-3. Skeletons and Numbering Schemes for the Classes of Flavonoids

Table 2-3. Spectral Data for Seven Compounds from Astragalus nuttallianus

Spot Number[1]	Color (UV)[2]	Color with NH_3 (UV)[2]	UV Absorbance Data[3] CH_3OH	NaOME[4]	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3
1	P	YG	271,290sh, 296sh,321	274,292sh, 360sh,396↑	275,299sh 346,384sh	276,298sh 342,382sh	268,333	268,328
2	B	BB	278,324	275,380↑	262,346	270,297sh 326	268,319	257,346
3	P	YG	258,265sh 354	281,423↑	269sh,273 426	269,297sh 362sh,404	261sh,268 366	261,268sh 379
4	P	YG	256,265sh 358	276,320m 415↑	275,303sh 438	269,298sh 360sh,406	259sh,267 365	261,377
5	P	YG	257,268sh 351	276,416↑	274,302sh 332m,432	269,298sh 360sh,406	261sh,268 364	261,377
6[5]	P	YG	257sh,266 348	275,325m 400↑	272,302sh 352sh,413	270,300sh 352,401	256sh,267 354	263,358
7	I	BB	280,312sh	280,334sh	274,308sh	273,308sh	279,312sh	277,314sh

[1] Reference Figure 2-3
[2] P = absorbent purple, B = blue, I = invisible, YG = yellow-green, BB = bright blue
[3] sh = shoulder, ↑ = increase in intensity relative to CH_3OH spectrum, m = minor absorbance band
[4] There was no decomposition for any of the NaOMe spectra
[5] Thin-layer chromatography indicates mixture of 3 compounds

Table 2-4. Suggested Structures for the Major Flavonoids in *Astragalus nuttallianus*

Spot Number[1]	Suggested Structure
1	5,4'-dihydroxy-6-methoxyflavone 7-glycoside
2	5-deoxy or 5-substituted, 3',4'-dihydroxyflavone 7-glycoside
3	5,7,3',4'-tetrahydroxyflavonol 3-glycoside
4	5,7,3',4'-tetrahydroxyflavonol 3-diglycoside
5	5,7,3',4'-tetrahydroxyflavonol 3-diglycoside
6[2]	flavonol 3-diglycoside
7[3]	structure not proposed

[1] Reference Figure 2-3
[2] Thin-layer chromatography indicates mixture of 3 compounds
[3] Probable nonflavonoid phenolic compound

data it can be concluded that the major flavonoid components of Astragalus nuttallianus are not isoflavonoids with the possible exception of Spot 7. However, many nonflavonoid phenolic compounds have Rf values similar to those for Spot 7 and although inconclusive, it is doubtful that this compound is an isoflavonoid. It should be noted that these data account for only those compounds present at high enough concentrations for isolation and UVS analysis from a small amount of plant material. The data do not preclude the presence of isoflavonoids in Astragalus nuttallianus as minor components. More conclusive data could be obtained through the analysis of a larger sample (~ 2 kg, dry weight), allowing the isolation and structural analysis of the full compliment of flavonoids present in Astragalus.

2.2.3 Quantitative Results

This section presents the quantitative results for the determinations of flavonoid concentrations in quail food plants at the Andrews and Bailey field sites. Comparisons of average flavonoid concentrations for the two sites are made along with comparisons of individual plant species. The data were examined to determine the following:

- How average flavonoid concentrations varied with moisture stress and time at the two sites,
- how flavonoid concentrations in individual species varied with moisture stress and time, and

- o if there was a significant difference in average flavonoid concentrations at the two sites and, if so, could this difference be related to moisture stress in the environment.

A total of 38 samples were analyzed by thin-layer chromatography (TLC)/in-situ absorbance densitometry using the techniques presented in Section 4.0. An additional 11 samples could not be quantitated because of poor TLC resolution and 2 samples were not analyzed because of insufficient material. Samples were quantitated according to a linear regression for qutin (5,6,3',4'-tetrahydroxyflavonol 3-rhamnoglucoside). Therefore, results are relative to rutin and do not represent absolute quantities. With current analytical techniques, absolute quantitation would have required isolation of the compounds for each sample. This was not feasible because of the large amount of plant material required, 1-2 kg, dry weight, and because of the level of effort required to isolate the flavonoids from 51 samples.

Because quantitation was accomplished relative to rutin, the reader should note that possible sources of error inherent in the analysis include response factors which may vary relative to the response for rutin and degrees of glycosylation, oxidation and methylation different from that of rutin. Because of these possible sources of additional error, the more conservative error estimate, the square root of the sum of the squares of the standard deviations for individual spot values, rather than the square root of the sum of the squares of the standard deviations divided by the number of spots was used to set error limits for individual samples.

Table 2-5 presents the quantitative results for each sample and the average values for each collection date and site. The values in this table were used to establish the correlations and conclusions discussed in the next section.

2.2.3.1 Average Concentration Correlations with Moisture Stress and Time

In order to determine if total flavonoid concentrations correlated with moisture and/or time in the environment, linear regressions were performed for flavonoid concentrations versus soil resistance at 4, 16 and 28 cm, average soil resistance and time for each site individually and for both sites combined. Soil moisture data, as resistance in μohms, for the two sites is presented in Table 2-6. Higher resistances indicate lower percentages of available moisture (0.5~100%, 1.0~72%, 2.0~50%, 15<20%). Only the readings for the collection dates were used for calculating linear regressions.

Correlations were determined using two different methods. First, each value for each collection date was given equal weighting and second, values for each date were weighted by a factor established by the number of individual samples which contributed to the value. The first technique was used on the premise that since each value represents what was available to the quail on a given date regardless of number of samples, equal weighting should be given to each collection date. However, statistically, it is preferable to use weighted correlations. Weighting expresses the level of confidence which can be attached to each data point

Table 2-5. Concentrations of Flavonoids in Quail Food Plants

Site	Date	Species	Flavonoid Concentration[1] (µg/g)
Andrews	01-22-81	Hymenopappus tenuifolius	2613 ± 60
		Oenothera albicaulis	235 ± 60
		Average	1424 ± 60
	02-10-81	Hymenopappus tenuifolius	7530 ± 1260[2]
	03-31-81	Allium drumondii	2347 ± 106
		Astragalus nuttallianus	1954 ± 94
		Lepedium virginicum	ND
		Lesquerella gordonii	153 ± 4.4
		Lithospermum incisum	1156 ± 170
		Average	1410 ± 221
	04-22-81	Ambrosia psilostachya	2630 ± 41[2]
		Astragalus nuttallianus	670 ± 19
		Descurainia richardsonii	2664 ± 41
		Lepidium virginicum	ND
		Lesquerella gordonii	883 ± 91
		Average	1405 ± 59
	05-31-81	Ambrosia psilostachya	225 ± 18
		Astragalus nuttallianus	422 ± 11
		Lepidium virginicum	ND
		Lesquerella gordonii	414 ± 60
		Lithospermum incisum	976 ± 20
		Average	509 ± 33
	06-02-81	Ambrosia psilostachya	364 ± 85
		Lesquerella gordonii	231 ± 24
		Lithospermum incisum	566 ± 74
		Average	384 ± 67

Table 2-5. (Cont.)

Site	Date	Species	Flavonoid Concentration[1] (µg/g)
Andrews (Cont.)	06-18-81	Ambrosia psilostachya	576 ± 29
		Lithospermum incisum	146 ± 21
		Average	361 ± 25
Bailey	01-14-81	Kochia scoparium	ND
		Machaeranthera sp.	154 ± 11
		Sphaeralcea coccinea	1980 ± 0.2
		Average	1067 ± 7.8
	03-05-81	Cryptantha mexicana	400 ± 24
		Eriogonum annuum	4210 ± 169
		Physalis viscosa	227 ± 6.5
		Average	1612 ± 99
	04-16, 17-81	Allium drumondii	1160 ± 51
		Ambrosia psilostachya	284 ± 34
		Astragalus nuttallianus	1297 ± 98
		Kochia scoparium	ND
		Lepidium virginicum	ND
		Lesquerella gordonii	1190 ± 8.6
		Lithospermum incisum	514 ± 34
		Machaeranthera sp	1880 ± 156
		Average	1054 ± 81
	05-08-81	Ambrosia psilostachya	646 ± 6.4
		Astragalus nuttallianus	ND
		Kochia scoparium	ND
		Lepidium virginicum	ND
		Lesquerella gordonii	ND
		Lithospermum incisum	306 ± 22
		Average	476 ± 16

Table 2-5. (Cont.)

Site	Date	Species	Flavonoid Concentration[1] (µg/g)
Bailey (Cont.)	05-21-81	Ambrosia psilostachya	725 ± 6.6
		Kochia scoparium	ND
		Lepidium virginicum	ND
		Lesquerella gordonii	153 ± 4.4
		Lithospermum incisum	256 ± 17
		Average	378 ± 11
	06-15-81	Ambrosia psilostachya	516 ± 11
		Kochia scoparium	ND
		Lithospermum incisum	1104 ± 23
		Average	810 ± 26

[1] All values relative to rutin
ND = Not Determined
[2] Value rejected at 99% confidence level

Table 2-6. Soil Resistance Data

Site	Date	Resistance at 4 cm (μohm)	Resistance at 16 cm (μohm)	Resistance at 28 cm (μohm)	Average Resistance (μohm)
Andrews	1-22*	0.8	0.8	0.8	0.8
	2-09	1.8	4.7	0.9	2.5
	2-25	5.4	1.5	1.0	2.6
	3-12	0.6	0.6	0.6	0.6
	3-31*	6.2	1.0	0.8	2.7
	4-22*	0.6	0.6	0.6	0.6
	5-12*	1.4	4.0	3.1	2.8
	6-02*	1.6	10.0	10.5	7.4
	6-19*	15.0	15.0	15.0	15.0
Bailey	1-14*	0.8	0.7	0.9	0.8
	2-02	3.5	3.3	3.7	3.5
	2-18	5.5	3.5	3.5	4.2
	3-04*	2.3	2.6	3.6	2.8
	3-17	0.6	4.2	4.3	3.0
	4-16*	4.7	3.3	3.7	3.9
	5-07*	4.3	4.6	5.5	4.8
	5-20*	14.8	14.8	12.8	14.1
	6-15*	15.0	13.8	14.8	14.5

* Correspond to collection dates

established by the number of samples contributing to that data point. That is, if six sample values contributed to the data point, there is a higher level of confidence in the point than if only two sample values contributed.

Tables 2-7 and 2-8 present the results of the unweighted and weighted linear regressions for the two sites treated both separately and combined. Two data points from the Andrews site, Hymenoppapus 02-10-81 and Astragalus 04-22-81, were rejected at the 99% confidence level using the F test for outliers (see Section 4.4) and were not used in establishing the correlations. Examples of the regression lines are shown in Figures 2-4 through 2-6. Figure 2-4 shows plots of average flavonoid concentrations versus average soil moisture for each site individually. Figure 2-5 plots flavonoid concentrations versus time for the two individual sites and Figure 2-6 shows the combined plots of flavonoid concentrations versus average soil moisture and versus time.

Negative correlation coefficients for concentration versus time and versus soil resistance (resistance indirectly proportional to moisture) indicate that flavonoid concentrations available in the quails' diet were depleted with time and a reduction of available soil moisture through the test period. Table 2-7 indicates that all values plotted showed negative unweighted correlation coefficients versus time and soil moisture. For the Andrews site all correlations were significant at the 95% confidence level except concentration versus resistance at 4 cm. The regression line for resistance at 16 and 28 cm and for average resistance have Y-intercepts and

Table 2-7. Unweighted Regression Values for Average Total Flavonoid Concentrations versus Soil Resistance and Time

Site	Parameter	Correlation Coefficient (r)	Y-Intercept (b)	Slope (m)	Data Points (n)	95% Confidence Level
Andrews	Resistance 4 cm	-0.386	1075	-37.3	6	0.621
	Resistance 16 cm	-0.860*	1327	-78.7	6	0.621
	Resistance 28 cm	-0.840*	1302	-75.0	6	0.621
	Average Resistance	-0.750*	1278	-74.2	6	0.621
	Time[1]	-0.819*	1876	- 8.50	6	0.621
Bailey	Resistance 4 cm	-0.586	1190	-41.6	6	0.621
	Resistance 16 cm	-0.614	1201	-45.6	6	0.621
	Resistance 28 cm	-0.554	1207	-44.7	6	0.621
	Average Resistance	-0.584	1202	-44.4	6	0.621
	Time[1]	-0.618	1417	- 5.11	6	0.621
Combined	Resistance 4 cm	-0.468*	1122	-38.2	12	0.457
	Resistance 16 cm	-0.740*	1270	-61.1	12	0.457
	Resistance 28 cm	-0.705*	1277	-63.6	12	0.457
	Average Resistance	-0.660*	1240	-56.8	12	0.457
	Time[1]	-0.714*	1618	- 6.63	12	0.457

[1] Time expressed in days. January 1 = Day 1

* Significant correlation at or above 95% confidence level

Table 2-8. Weighted Regression Values for Average Total Flavonoid Concentrations versus Soil Resistance and Time

Site	Parameter	Correlation Coefficient (r)	Y-Intercept (b)	Slope (m)	Data Points (n)	95% Confidence Level
Andrews	Resistance 4 cm	-0.203	1008	-22.3	6	0.621
	Resistance 16 cm	-0.831*	1307	-83.0	6	0.621
	Resistance 28 cm	-0.801*	1271	-77.5	6	0.621
	Average Resistance	-0.688*	1257	-77.4	6	0.621
	Time[1]	-0.807*	2015	- 9.52	6	0.621
Bailey	Resistance 4 cm	-0.668*	1278	-50.6	6	0.621
	Resistance 16 cm	-0.680*	1261	-51.9	6	0.621
	Resistance 28 cm	-0.620*	1278	-52.9	6	0.621
	Average Resistance	-0.642*	1253	-59.5	6	0.621
	Time[1]	-0.645*	1560	- 6.05	6	0.621
Combined	Resistance 4 cm	-0.393	1113	-34.9	12	0.457
	Resistance 16 cm	-0.942*	1282	-64.8	12	0.457
	Resistance 28 cm	-0.704*	1277	-63.6	12	0.457
	Average Resistance	-0.644*	1254	-60.1	12	0.457
	Time[1]	-0.737*	1769	- 7.69	12	0.457

[1] Time expressed in days. January 1 = Day 1

* Significant correlation at or above 95% confidence level

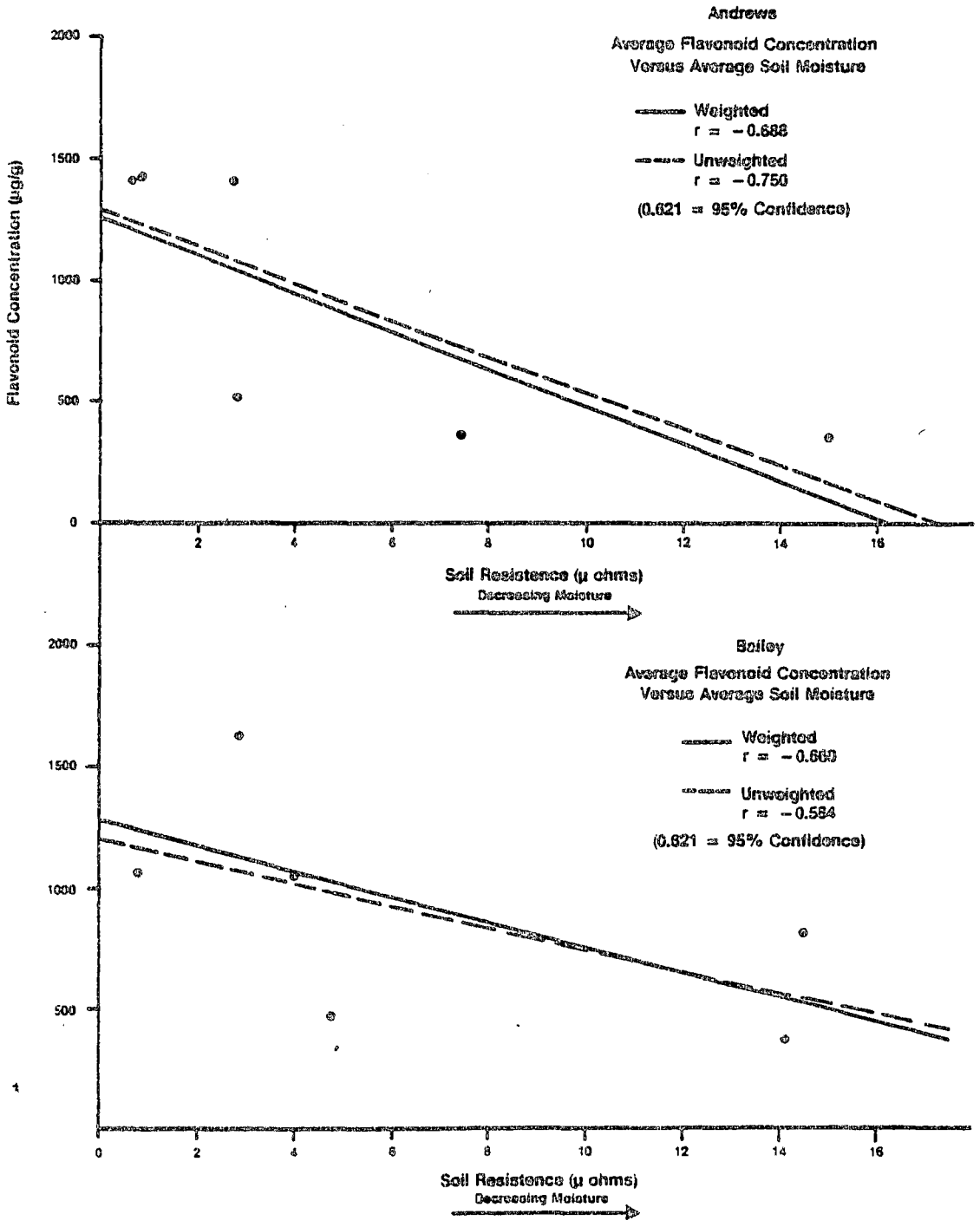


Figure 2-4. Regression Lines for Average Flavonoid Concentrations versus Average Soil Moisture for the Andrews and Bailey Test Sites

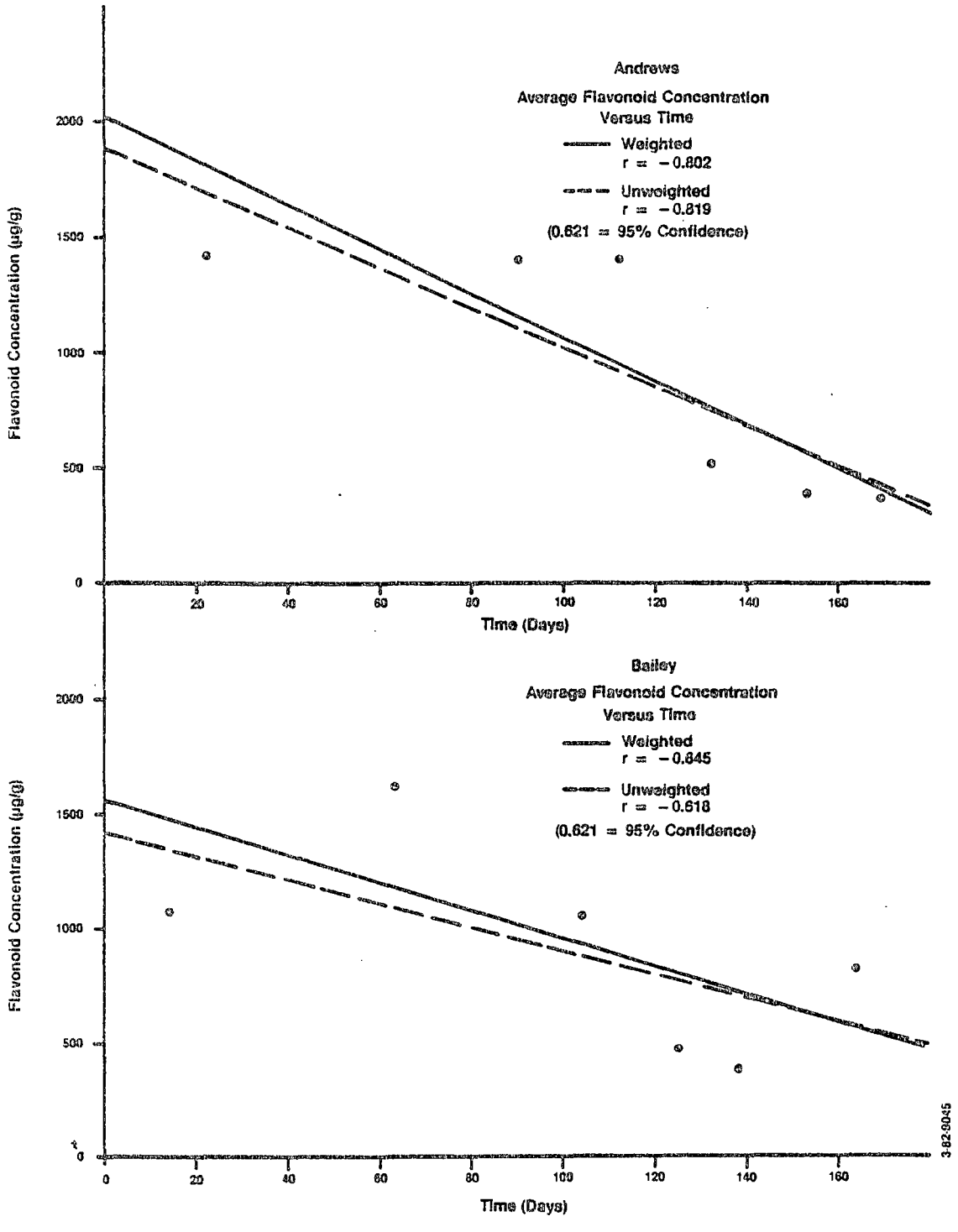


Figure 2-5. Regression Lines for Average Flavonoid Concentrations versus Time for the Andrews and Bailey Test Sites

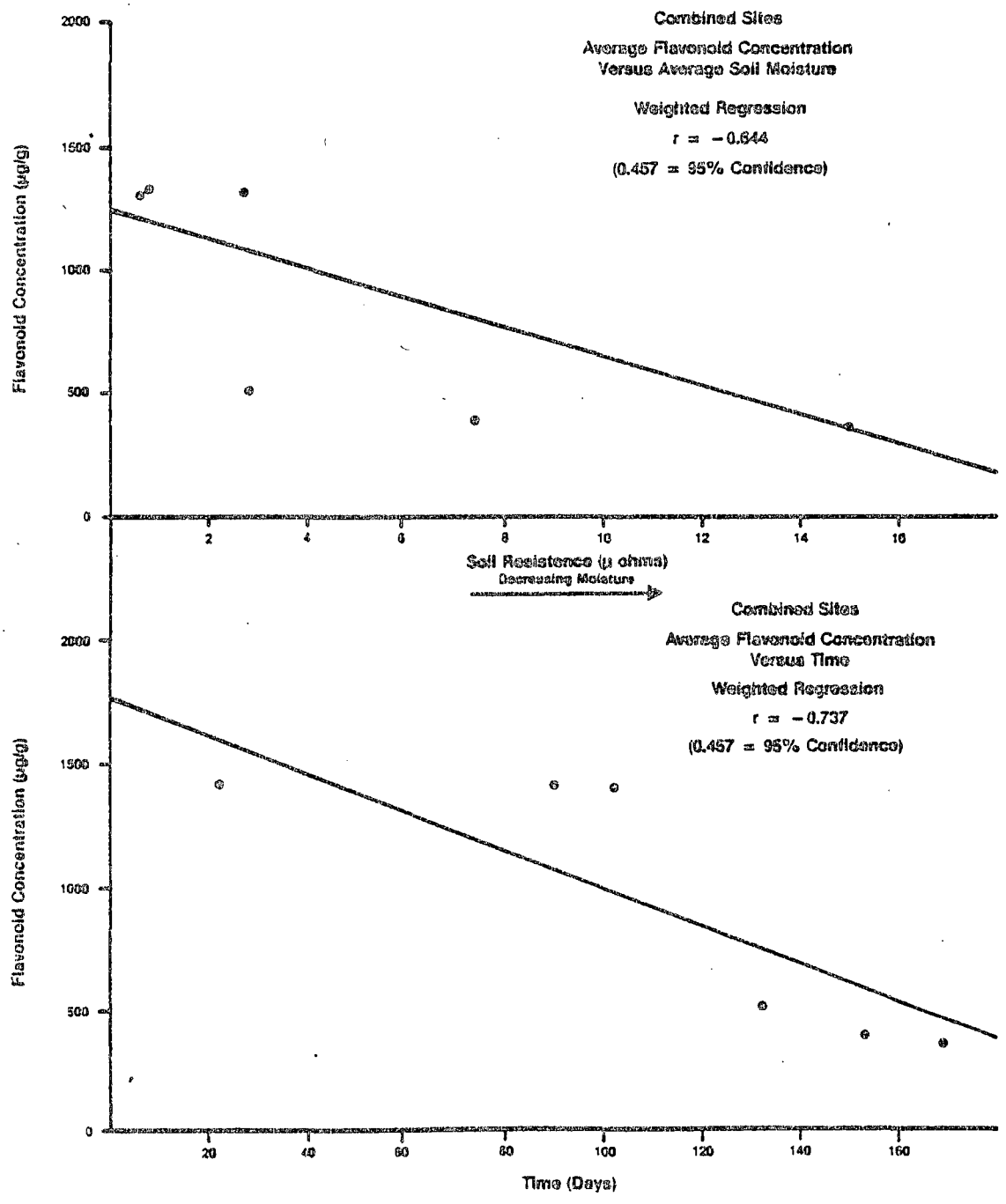


Figure 2-6. Regression Lines for the Combined Data for Average Flavonoid Concentrations versus Average Soil Moisture and Time

slopes indicating no significant difference in concentration response to these variables. Table 2-8 shows that these observations are also true for the weighted regression values. For the Bailey site, although no correlation coefficient was significant at the 95% confidence level for the unweighted data, all coefficients for all parameters showed negative correlations approaching the 95% confidence level. When the data were weighted (Table 2-8), all coefficients for the Bailey site for all parameters showed negative correlations at the 95% confidence level. When the values were combined for the sites these negative correlations still held and both unweighted and weighted coefficients were significant at the 95% confidence level for all parameters except the weighted regression line for resistance at 4 cm.

These data indicate that for both sites, as the season progresses from January to June, and the environments of the sites become drier, the average concentrations of flavonoids in the quails' diet are reduced. It should be emphasized that these data do not conclude that the concentration in a specific tissue for a given species is reduced with decreased moisture. While this may play a role in the negative correlations, other factors such as tissue type and maturity (i.e., leaf tissue early in the season versus stem and seed tissue late) and the presence of different plant species in the environment may also play important roles along with other variables which may not have been taken into consideration. Observations on individual species are made in the following section.

2.2.3.2 Individual Species Correlations

In addition to the establishment of correlations for average flavonoid concentrations versus moisture stress, a number of individual species were examined. In these cases, analagous tissues were analyzed when possible. The individual species which were examined for moisture stress correlations were:

- o Ambrosia psilostachya: Andrews site, Bailey site and combined;
- o Astragalus nuttallianus: Andrews site and combined;
- o Lesquerella gordonii: Andrews site and combined; and
- o Lithospermum incisum: Andrews site, Bailey site and combined.

Flavonoid concentrations in each species were plotted versus soil resistance at 4, 16 and 28 cm, average soil resistance and time. Unfortunately these correlations could not be performed for Kochia scoparium and Lepidium virginicum because poor thin-layer resolution made densitometric analysis of these species impossible.

The regression values for Ambrosia are given in Table 2-9. Figure 2-7 illustrates the plots of Ambrosia flavonoid concentrations versus time and average soil resistance for each site individually. Unlike the average concentrations of flavonoids, Ambrosia showed positive correlations for reduced soil moisture and increasing time. In the case of the

Table 2-9. Regression Values for Flavonoid Concentrations in *Ambrosia psilostachya* versus Soil Resistance and Time

Site	Parameter	Correlation Coefficient (r)	Y-Intercept (b)	Slope (m)	Data Points (n)	95% Confidence Level
Andrews	Resistance 4 cm	0.924*	262	21.0	3	0.805
	Resistance 16 cm	0.985*	82.7	31.6	3	0.805
	Resistance 28 cm	0.982*	118	29.0	3	0.805
	Average Resistance	0.997*	148	28.7	3	0.805
	Time[1]	0.980*	-1025	9.34	3	0.805
Bailey	Resistance 4 cm	0.416	409	13.6	7	0.729
	Resistance 16 cm	0.560	379	18.0	4	0.729
	Resistance 28 cm	0.498	388	16.6	4	0.729
	Average Resistance	0.493	388	16.6	4	0.729
	Time[1]	0.465	68.1	3.58	4	0.729
Combined	Resistance 4 cm	0.666*	322	19.3	7	0.582
	Resistance 16 cm	0.617*	271	22.0	7	0.582
	Resistance 28 cm	0.599*	273	21.8	7	0.582
	Average Resistance	0.659*	272	23.0	7	0.582
	Time[1]	0.330	101	2.69	7	0.582

[1] Time expressed in days. January 1 = Day 1

* Significant correlation at or above 95% confidence level

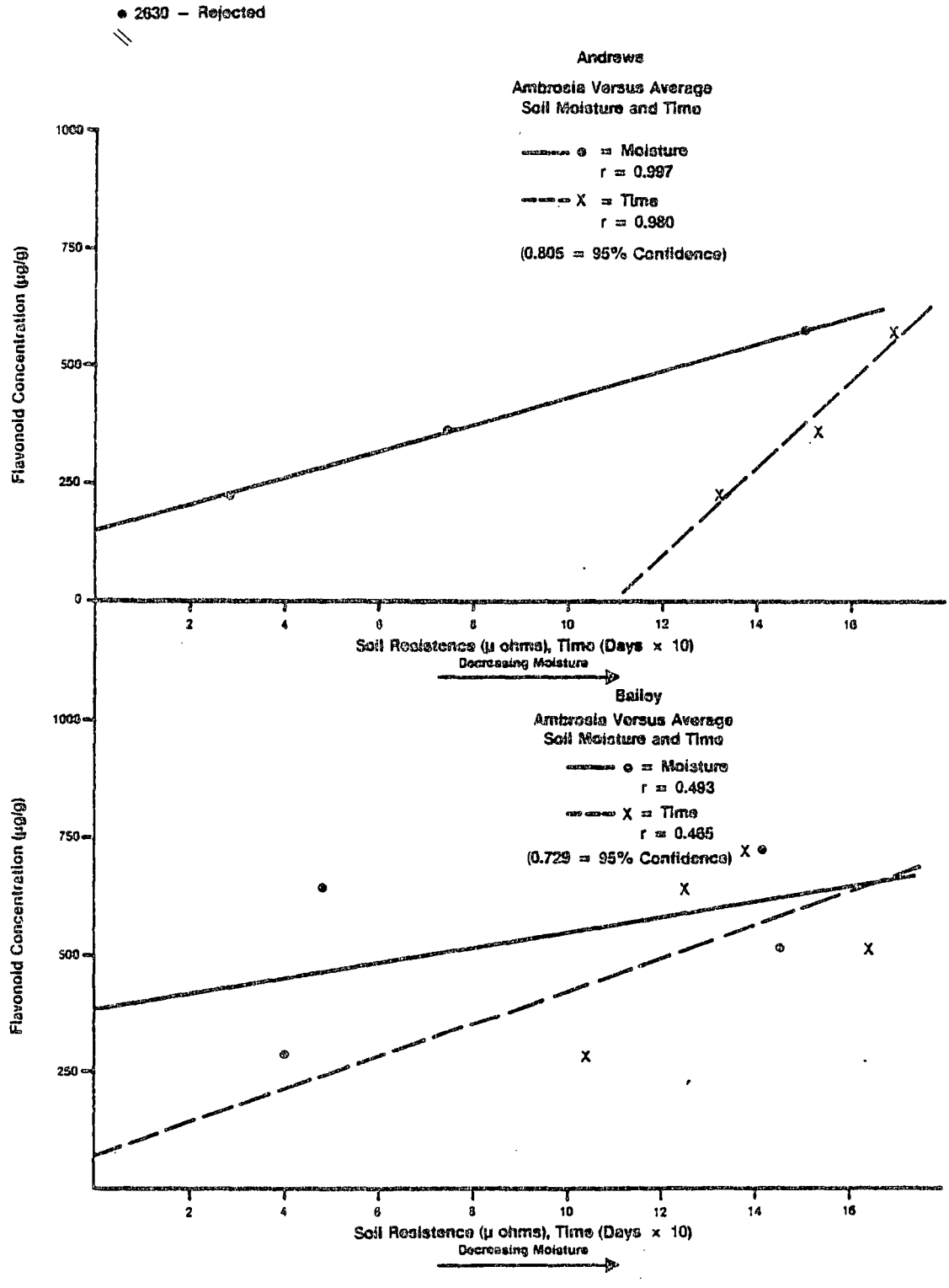


Figure 2-7. Regression Lines for Flavonoid Concentrations in Ambrosia psilostachya versus Average Soil Moisture and Time

Andrews site, these correlations were above the 95% confidence level for time and soil resistance at all depths. This was also true of the combined site plot with the exception of concentration versus time. While no parameter at the Bailey site showed correlations at the 95% confidence level, all correlations were positive, again indicating an increase in flavonoid concentration with a decrease in moisture.

The regression values for Astragalus at the Andrews site and the combined site values are given in Table 2-10 and plotted for the Andrews site in Figure 2-8. As shown, the only significant correlations for both the Andrews data and the combined data were for concentration versus resistance at 4 cm and versus time.

The correlations versus time were negative suggesting a decrease in concentration versus increasing time. However, the correlations versus resistance at 4 cm were positive indicating an increase in concentration versus decreasing soil moisture at that depth. Correlations versus soil resistance at 16 and 28 cm were negative while correlations versus average soil resistance were positive. None of these correlations were significant at the 95% confidence level. While these data indicate that Astragalus responds to soil moisture availability at 4 cm and to time, more data are needed to positively establish that the concentrations are not affected by soil moisture at greater depths.

The regression values for Lesquerella at the Andrews site and the combined site values are given in Table 2-11 and plotted in Figure 2-8.

Table 2-10. Regression Values for Flavonoid Concentrations in *Astragalus nuttallianus* versus Soil Resistance and Time

Site	Parameter	Correlation Coefficient (r)	Y-Intercept (b)	Slope (m)	Data Points (n)	95% Confidence Level
Andrews	Resistance 4 cm	0.960*	303	261	3	0.805
	Resistance 16 cm	-0.537	1459	-238	3	0.805
	Resistance 28 cm	-0.569	1512	-324	3	0.805
	Average Resistance	0.326	577	216	3	0.805
	Time[1]	-0.941*	5117	- 36.8	3	0.805
Combined	Resistance 4 cm	0.952*	295	247	4	0.729
	Resistance 16 cm	-0.397	1428	-152	4	0.729
	Resistance 28 cm	-0.284	1341	-124	4	0.729
	Average Resistance	0.372	630	181	4	0.729
	Time[1]	-0.944*	5127	- 36.9	4	0.729

[1] Time expressed in days. January 1 = Day 1
* Significant correlation at or above 95% confidence level

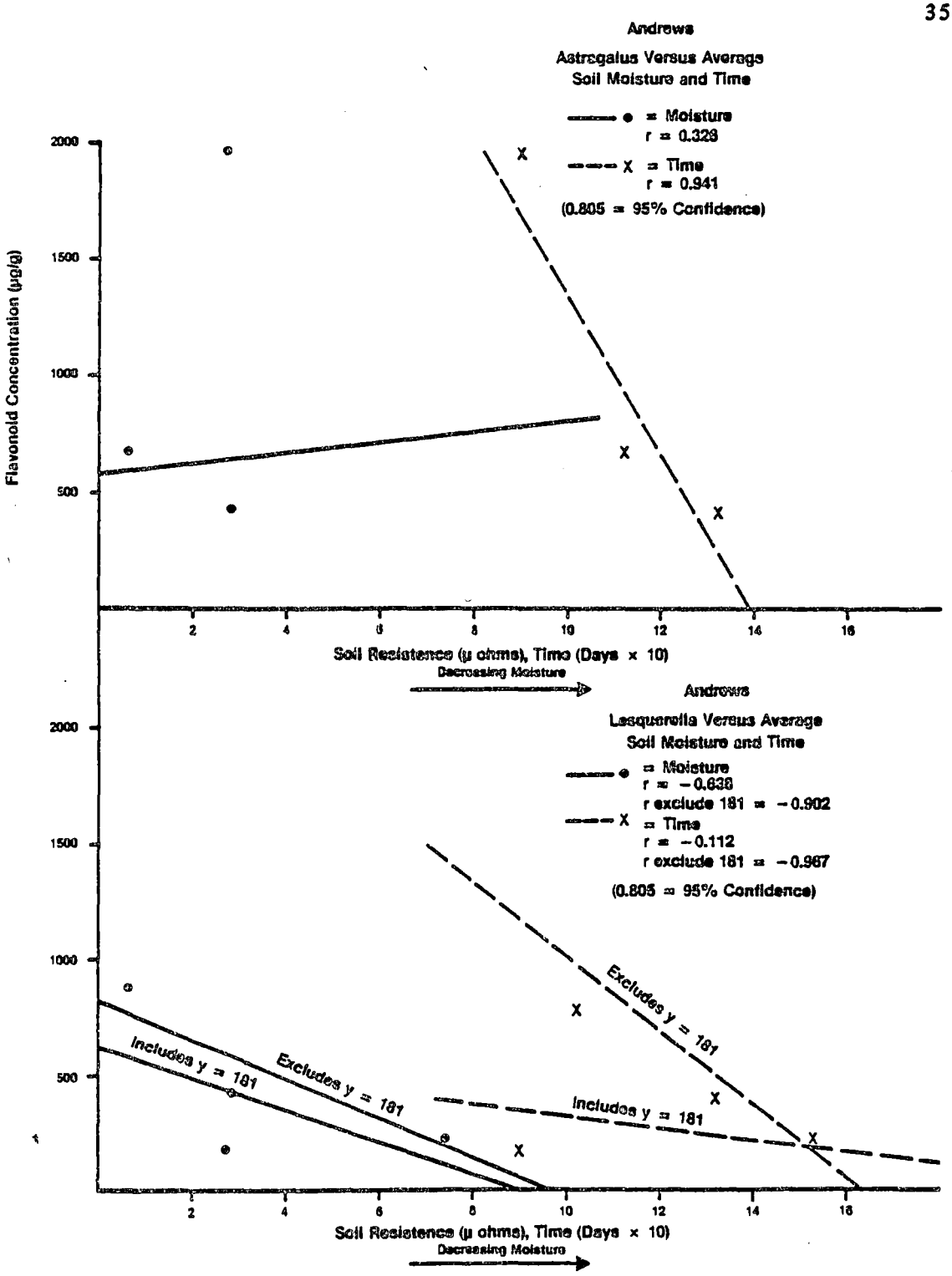


Figure 2-8. Regression Lines for Flavonoid Concentrations in Astragalus nuttallianus and Lesquerella gordonii

Table 2-11. Regression Values for Flavonoid Concentrations in *Lesquerella gordonii* versus Soil Resistance and Time

Site	Parameter	Correlation Coefficient (r)	Y-Intercept (b)	Slope (m)	Data Points (n)	95% Confidence Level
Andrews	Resistance 4 cm	-0.651	628	-82.1	4	0.729
	Resistance 16 cm	-0.477	564	-35.2	4	0.729
	Resistance 28 cm	-0.456	547	-31.5	4	0.729
	Average Resistance	-0.683	683	-76.6	4	0.729
	Time[1]	-0.112	589	- 1.32	4	0.729
Combined	Resistance 4 cm	-0.392	663	-31.7	6	0.621
	Resistance 16 cm	-0.519	732	-39.7	6	0.621
	Resistance 28 cm	-0.485	722	-40.5	6	0.621
	Average Resistance	-0.500	740	-44.1	6	0.621
	Time[1]	-0.410	1419	- 7.48	6	0.621

[1] Time expressed in days. January 1 = Day 1
* Significant correlation at or above 95% confidence level

Flavonoid concentrations showed no correlations at the 95% confidence level. However, all correlations were negative suggesting a decrease in concentration with increasing time and decreasing moisture. In addition, although the value for Andrews 03-31-81 (181 µg/g) cannot be rejected at the 95% confidence level, the value does not appear to fit with the other values for Lesquerella. If regression lines are plotted for the data points with this value excluded, the negative correlations become significant at the 95% confidence level for soil resistance at all depths and for time for both the Andrews site and combined sites with the exception of concentration versus resistance at 4 cm for the combined data points. The regression lines for the Andrews site with this data point excluded are also plotted in Figure 2-8.

The regression values for Lithospermum for the Andrews and Bailey sites are given in Table 2-12 and plotted for the individual sites in Figure 2-9. For the Andrews site, negative correlations were seen at well above the 95% confidence level for resistance at 16 and 28 cm, average resistance and time. This indicates a decrease in flavonoid concentrations with increasing time and decreasing soil moisture. The data for the Bailey site were inconclusive. In opposition to the Andrews site, these data showed positive correlations with resistance and time. However, no correlation for the Bailey site was significant at the 95% confidence level. If the data are plotted excluding the apparent outlier (Bailey 06-15-81, 1104 µg/g) the data show stronger negative correlations than the positive correlations seen with the point included. However, these negative correlations are not significant at the 95% confidence level except for concentrations

Table 2-12. Regression Values for Flavonoid Concentrations in *Lithospermum incisum* versus Soil Resistance and Time

Site	Parameter	Correlation Coefficient (r)	Y-Intercept (b)	Slope (m)	Data Points (n)	95% Confidence Level
Andrews	Resistance 4 cm	-0.648	989	-45.8	4	0.729
	Resistance 16 cm	-0.995*	1252	-71.0	4	0.729
	Resistance 28 cm	-0.994*	1215	-68.3	4	0.729
	Average Resistance	-0.967*	1223	-75.4	4	0.729
	Time[1]	-0.923*	2363	-12.1	4	0.729
Bailey	Resistance 4 cm	0.418	283	27.0	4	0.729
	Resistance 16 cm	0.319	357	20.6	4	0.729
	Resistance 28 cm	0.494	222	35.2	4	0.729
	Average Resistance	0.414	284	28.0	4	0.729
	Time[1]	0.638	-0.768	9.89	4	0.729
Combined	Resistance 4 cm	-0.224	744	-14.7	8	0.549
	Resistance 16 cm	-0.419	871	-29.0	8	0.549
	Resistance 28 cm	-0.394	858	-27.8	8	0.549
	Average Resistance	-0.366	839	-26.6	8	0.549
	Time[1]	-0.294	1194	-4.2	8	0.549

[1] Time expressed in days. January 1 = Day 1

* Significant correlation at or above 95% confidence level

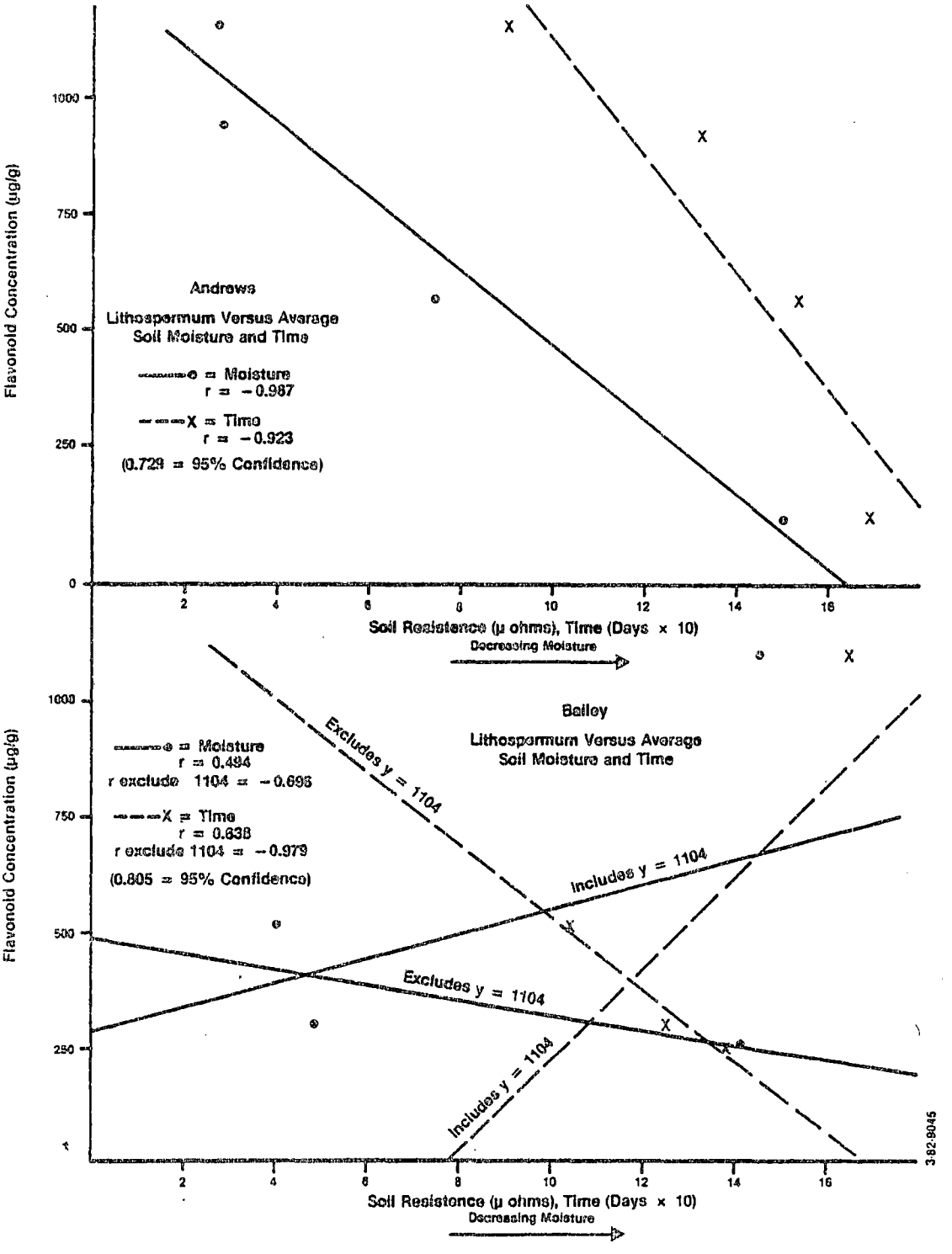


Figure 2-9. Regression Lines for Flavonoid Concentrations in Lithospermum incisum versus Average Soil Moisture and Time

versus time. Again, the correlations for the combined data are inconclusive. Although all correlations are negative, none are significant at the 95% confidence level. But in this case, if the apparent outlier (Bailey 06-15-81) is excluded, the negative correlations for resistance at 16 and 28 cm, average resistance and time become significant at the 95% confidence level. From these data, it is concluded that flavonoid concentrations at the Andrews site decreased with decreasing moisture and increasing time. However, the data are inconclusive for the Bailey site and the combined sites, although they suggest a response similar to the Andrews site response.

In summary, intraspecific response to soil resistence and time varied with the species examined. Ambrosia flavonoid concentrations increased with decreasing soil moisture and with time. Astragalus flavonoid concentrations decreased with decreasing soil moisture at 4 cm and with increasing time. Lithospermum and apparently Lesquerella concentrations decreased with decreasing soil moisture.

2.2.3.3 Intersite Comparisons of Quantitative Results

In order to compare intersite flavonoid concentrations, comparisons were made of average total flavonoid concentrations over equivalent time periods and of individual species concentrations at the two sites. These data were then compared to soil resistance data for the two sites.

In order to make the average total flavonoid comparisons, mean and weighted mean values (see Section 2.2.3.1 for rationale) for the two sites were calculated for the total time periods for both sites. In addition, values were calculated for the time period from mid-January through mid-April when the two sites showed relatively low soil resistence values (relatively high available moisture), and for the period from mid-May through mid-June when the two sites showed relatively high soil resistance values (relatively low available moisture).

The concentration data for these time periods are given in Table 2-13 and the corresponding soil resistance data in Table 2-14. As shown in Table 2-13, the average flavonoid concentrations for the entire test period for the two sites were virtually identical. When the values for the relatively wet period from mid-January through mid-May are compared, the flavonoid concentrations are similar with the Andrews site showing slightly higher concentrations. During this period, the Andrews site also showed lower moisture resistance values (Table 2-14) indicating a higher percentage of available moisture. These data appear to support the correlations discussed in Section 2.3.3.1, where, in general, average total flavonoid concentrations decreased with decreasing soil moisture in the environments of the two sites. The values from the period of mid-May through mid-June from the Andrews site showed lower average concentrations of flavonoids. However, in this case average soil resistance values were also lower indicating more available moisture. These data apparently contradict the moisture-concentration relations indicated for the mid-January through mid-

Table 2-13. Average Total Flavonoid Concentrations for the Andrews and Bailey Sites for Comparable Time Periods

Site	Time Period	Average Flavonoid Concentration[1] (Unweighted)	Average Flavonoid Concentration[1] (Weighted)
Andrews	01/22 - 06/18	915 ± 66[2]	923 ± 70[2]
Bailey	01/14 - 06/15	900 ± 54	944 ± 70
Andrews	01/22 - 04/22	1413 ± 80	1411 ± 90
Bailey	01/14 - 04/16	1244 ± 74	1208 ± 81
Andrews	05/12 - 06/18	418 ± 47	424 ± 15
Bailey	05/09 - 06/15	559 ± 19	529 ± 17

[1] Relative to rutin
[2] Error limits reflect estimation of analytical error, not σ of mean values

Table 2-14. Average Soil Resistance Values for the Andrews and Bailey Sites for Comparable Time Periods

Site	Time	Soil Resistance (μohm)[1] 4 cm	16 cm	28 cm	Average
Andrews	01/22 - 06/18	3.7	4.2	3.7	3.9
Bailey	01/14 - 06/15	5.7	5.6	5.8	5.7
Andrews	01/22 - 04/22	2.6	1.5	0.8	1.6
Bailey	01/14 - 04/16	2.9	2.9	3.2	3.1
Andrews	05/12 - 06/18	6.0	9.7	9.6	8.4
Bailey	05/09 - 06/15	11.0	11.0	11.0	11.0

[1] Soil resistance and available moisture are inversely proprtional.

April. However, if the apparent outlier (Bailey, Lithospermum, 06-15-81) is excluded from the Bailey average for this time period, the mean concentration changes to 457 ± 13 and the weighted mean to 433 ± 16. Both values are statistically equivalent to the Andrews mean and weighted mean. It is difficult to conclude that there is any significant difference in flavonoid concentrations at the two sites from the data presented in Table 2-13.

Comparisons of flavonoid concentrations for individual species at the two sites are shown in Table 2-15. This table presents average concentrations for all samples of Ambrosia, Astragalus, Lesquerella and Lithospermum from the two sites and for only those samples from comparable time periods. Although there is considerable variation in the individual species comparisons, differences are reduced when only those samples from comparable time periods are considered. The flavonoid concentrations from both Lesquerella and Lithospermum from the two sites from comparable time periods are statistically equivalent. Although the Astragalus samples from comparable time periods showed a significant concentration difference, this comparison is based on only a single sample from each site. The concentration of flavonoids in Ambrosia from comparable time periods was higher for the Bailey site (629 ± 8.3 versus 388 ± 53). However, these concentrations are still fairly similar. The Bailey site also showed higher soil resistance (lower available moisture) for the time period in which the Ambrosia samples were taken. This would apparently support the positive correlations observed for flavonoid concentrations in Ambrosia versus soil resistance (see Section 2.2.3.2).

Table 2-15. Average Flavonoid Concentrations for Individual Species at the Andrews and Bailey Sites

Species	Site	Average Flavonoid Concentration[1] (μg/g)	Collection Dates	Concentrations for Comparable Time Periods[1] (μg/g)	Collection Dates
Ambrosia psilostachya	Andrews	388 ± 53[2]	5/13,6/2,6/18	388 ± 53[2]	5/13,6/2,6/18
	Bailey	542 ± 19	4/16,5/8,5/20,6/15	629 ± 8.3	5/8,5/20,6/15
Astragalus nuttallianus	Andrews	1015 ± 56	3/31,4/22,5/12	670 ± 19	4/22
	Bailey	1297 ± 98	4/16	1297 ± 98	4/16
Lesquerella gordonii	Andrews	427 ± 56	3/31,4/22,5/12,6/2	652 ± 77	4/22,5/12
	Bailey	672 ± 6.8	4/16,5/20	672 ± 6.8	4/16,5/20
Lithospermum incisum	Andrews	711 ± 94	3/31,5/13,6/2,6/18	559 ± 46	5/13,6/2,6/18
	Bailey	545 ± 25	4/16,5/8,5/20,6/15	555 ± 21	5/8,5/20,6/15

[1] Relative to rutin
[2] Error limits reflect estimation of analytical error, not σ of mean values

2.3 General Conclusions and Recommendations

This section presents the general conclusions and recommendations based on the results of this study.

2.3.1 General Conclusions

The following general conclusions are made based on the results of this study presented in Section 2.2:

- It is highly unlikely that isoflavonoids comprise a significant component of the flavonoid concentrations in the diets of quail from the two west Texas sites. From paper chromatographic data and the literature it was concluded that *Astragalus nuttallianus* was the only species likely to contain isoflavonoids. Ultraviolet spectrophotometric data show that the major flavonoids in this species are not isoflavonoids.
- Average total flavonoid concentrations at both sites showed negative correlations with soil resistance and time indicting decreased levels of flavonoids in the quails' diet with decreasing moisture. This may be due to a combination of moisture stress, variation in species present, variations in tissue types or possibly other factors which have not been taken into consideration.

- o Individual species vary in response to soil resistance and time. Ambrosia psilostachya showed significant positive correlations while Astragalus nuttalianus, Lesquerella gordonii and Lithospermum incisum all showed negative correlations at varying levels of significance.
- o There is no clear indication of intersite variation in average total flavonoid concentrations present in the diets of quail.

2.3.2 Recommendations

Based on the results of this study a number of recommendations are made. These include both recommendations for analytical procedure modification and for further investigations in the role of flavonoids in quail reproduction.

2.3.2.1 Analytical Recommendations

It may be possible to improve analytical methods to reduce the errors introduced by variations in the absorbance characteristics of individual flavonoids. To accomplish this, a method other than spectrophotometric determination is preferred. An obvious analytical method which would provide a uniform or easily corrected response for flavonoids is gas chromatography with flame ionization detection (GC-FID). GC-FID responds to the number of carbon atoms present in the molecule rather than its

absorption characteristics. However, there are problems which must be solved before this approach could be considered viable. Because flavonoids are relatively high molecular weight compounds with high degrees of hydroxylation and varying glycosylation, chromatographing the compounds is difficult. First, the compounds must be hydrolyzed to remove the sugar moieties. Even after hydrolysis, the highly polar nature of these compounds has made methylation of the free hydroxyl groups necessary for GC analysis. However, it may be possible to chromatograph flavonoids without methylation using modern chromatographic techniques. It is recommended that on-column injection through a cool injection port using a short capillary column be evaluated for flavonoid analysis. If the compounds can be chromatographed directly using this technique, the obvious advantages would be a uniform response factor by FID detection and the elimination of the need for methylation.

If direct analysis by GC-FID is not possible, the utility of quantitation after methylation should be evaluated. It is known that the methyl derivatives of flavonoids can decompose over time under normal laboratory conditions. However, careful handling might greatly reduce this problem. This would include taking care to keep the methylated samples cold and not allowing exposure to light. In addition, if the decomposition is a result of oxidation, performing the laboratory procedures in an inert gas atmosphere and blanketing the methylated compounds with an inert gas might significantly reduce decomposition rates. Alternatively, it might be possible to form other derivatives which are more stable than the methyl derivatives.

In either case, an inherent problem in GC-FID analysis is the lack of qualitative information furnished by the analysis. If either analytical technique proves successful, this problem is easily solved by gas chromatography-mass spectrometry (GS-MS) analysis . GC-MS could be used with GC-FID to provide qualitative data. To accomplish this, one sample from each species would be analyzed by GC-MS using exactly the same chromatographic conditions used for the GC-FID analyses. Structural data from GC-MS could then be related to GC-FID retention times. This GC-MS analysis could then be related to other samples of the same species, eliminating the need to perform a GC-MS analysis on each sample.

If neither GC technique proved satisfactory for quantitation, another possibility is the use of an alternative spray reagent for development of flavonoids on thin-layer plates. While Natural Products Reagent A has the advantage of greatly increasing the sensitivity of thin-layer analysis, it has the disadvantage of merely shifting the absorption bands of the compounds. Flavonoid absorptions must still be measured with the analytical error introduced from varying maximum absorbance wavelengths and intensities. If a spray reagent could be obtained or developed which reacted quantitatively with the flavonoids and at the same time had its own characteristic absorption independent of and resolved from the flavonoids, this could provide a uniform response factor for densitometric analysis.

2.3.2.2 Recommendations for Further Study

While the results of the current study indicate that flavonoids are probably not a major factor in quail reproductive fecundity, prior to eliminating the compounds as factors in reproduction, the following recommendations are made:

- Since isoflavonoids may be oestrogenic at relatively low concentrations, it is recommended that the flavonoid chemistry of Astragalus nuttallianis be further examined to determine if isoflavonoids are present as relatively minor compounds in relation to the known compounds present.
- Feeding experiments under controlled conditions with isoflavonoids should be conducted to determine if these compounds are oestrogenic in avian metobolism.
- The current study should be continued to determine whether or not the environmental factors contributing to flavonoid concentrations in the diet were sufficiently different at the two sites to cause significant differences. The addition of a third site with more contrasting environmental conditions might serve to enhance any correlations.
- It is possible that other natural products present in the quails' diet may affect their reproduction. Therefore, it is recommended that a thorough literature search be conducted to

determine any other compounds which might be related to reproductive fecundity. If likely candidate compounds are found, quail food plants could be screened for these compounds with subsequent quantitation if detected.

3.0 SAMPLING

Samples for this project were collected by United States Department of Agriculture (USDA) Forest Service personnel. Samples were collected from two sites in west Texas. The Andrews County site is located approximately eight miles northwest of Andrews, Texas. Collections were made at this site over the period of January 22, 1981, to June 18, 1981. The Bailey County site is located approximately 15 miles southwest of Muleshoe, Texas. Collections at the Bailey site were made over the period of January 14, 1981, to June 15, 1981. Quail were collected concurrently with plant specimens from the two sites to determine food habits. Plant species in the diet were determined in the field, and the major food plants were collected for subsequent analysis.

The plant specimens were initially dried by USDA personnel and voucher specimens were prepared for each collection. Selected vouchers were sent to the author along with the bulk samples for confirmation of sample identifications. Final identifications were made by the author at the Herbarium of The University of Texas at Austin by comparison to representative reference specimens on file at the herbarium collected from the same areas in west Texas. Table 3-1 presents the food plants which were collected at each site, the dates of collection and the bulk tissue collected for subsequent analysis. As Table 3-1 illustrates, a total of 51 samples were collected, 23 from the Andrews site collected on 7 dates and

Table 3-1. Quail Food Plants Collected from Two Sites in West Texas

Site	Date	Plant Species	Tissue[1]
Andrews	01-22-81	Hymenopappus tenuifolius	Le,St
		Oenothera albicaulis	Le
	02-10-81	Hymenopappus teniufolius	Le,St
	03-31-81	Allium drummondii	Le,St,Fl
		Astragalus nuttallianus	Le
		Lepidium virginicum	Le,St
		Lesquerella gordonii	Le,St,Fl
		Lithospermum incisum	Le,St
	04-22-81	Ambrosia psilostachya	Le
		Astragalus nuttallianus	Le,St,Se
		Descurainia richardonsii	Le,St
		Lepidium virginicum	St,Se
		Lesquerella gordonii	Le,St,Se
	05-13-81	Ambrosia psilostachya	Le
		Astragalus nuttallianus	Le,St,Se
		Lepidium virginicum	St,Se
		Lesquerella gordonii	Le,St,Se
		Lithospermum incisum	Le,Se
	06-02-81	Ambrosia psilostachya	Le
		Lesquerella gordonii	Le,Fl,Se
		Lithospermum incisum	Le,St,Se
	06-18-81	Ambrosia psilostachya	Le
		Lithospermum incisum	Le
Bailey	01-14-81	Kochia scoparium	Fl,Se
		Machaeranthera sp.[2]	Le,St
		Sphaeralcea coccinea	Le
	03-05-81	Cryptantha mexicana	Le
		Eriogonum annuum	Le
		Physalis viscosa	Le

Table 3-1. (Cont.)

Site	Date	Plant Species	Tissue[1]
Bailey (Cont.)	04-16,17-81	Allium drumondii	Le,St,Fl
		Ambrosia psilostachya	Le
		Astragalus nuttallianus	Le,St
		Kochia scoparium	Le
		Lepidium virginicum	Le,St
		Lesquerella gordonii	Le,St
		Lithospermum incisum	Le,St
		Machaeranthera sp.[2]	Le,St
	05-08-81	Ambrosia psilostachya	Le
		Astragalus nuttallianus	insufficient[3]
		Kochia scoparium	Le
		Lepidium virginicum	St,Se
		Lesquerella gordonii	insufficient[3]
		Lithospermum incisum	Le,St,Se
	05-21-81	Ambrosia psilostachya	Le
		Kochia scoparium	Le,St
		Lepidium virginicum	St,Se
		Lesquerella gordonii	Le,St,Se
		Lithospermum incisum	Le,Se
	06-15-81	Ambrosia psilostachya	Le
		Kochia scoparium	Le
		Lithospermum incisum	Le

[1] Le = Leaves, St = Stems, Fl = Flowers, Se = Seeds
[2] Species not determined; voucher specimen not examined
[3] Insufficient material for analysis

28 from the Bailey site collected on 6 dates. These samples represented 15 genera in 11 families. Also, as indicated in the table, the tissue types differed from sample to sample and variously included one or more types including leaves, stems, flowers and seeds.

In most cases, sampling dates for the two sites were established so that samples were collected as concurrently as possible from each site. With three exceptions, collections were made at one site within approximately one week of collections at the other site. The exceptions were the February 10 and March 31 Andrews collections and the March 5 Bailey collection.

In addition to the collection of plant samples, moisture in the environments of the two sites was monitored approximately every two weeks by USDA personnel. There determinations were made at five stations for each of the two sites. Accumulated winter rainfall was determined up to the initial collection date for each site. After the first collection date, accumulated rainfall was determined for each approximate two-week interval. Soil moisture determinations were also made at each station for each site by measuring soil resistance at depths of 4, 16 and 28 centimeters. Biweekly values for rainfall and soil moisture at each of the depths were determined by averaging the five station values for each of the four parameters. In all cases, a biweekly determination was made within 24 hours of the plant collection dates.

4.0 ANALYTICAL METHODS

The analytical methods used for the completion of the project included plant extraction and extract clean up, two-dimensional paper chromatography, two-dimensional thin-layer chromatography, scanning ultraviolet spectrophotometry and scanning absorbance densitometry. An analytical flow diagram is shown in Figure 4-1. The analytical methods that were used are discussed below.

4.1 Plant Extraction

Plant extractions were carried out using techniques similar to those of Wilkins and Bohm (Ref. 10). Samples were received as dried material and further dried for 48 hours at 60° C to remove residual moisture and allow the determination of an accurate dry weight. When sufficient material was available, 10 g of the dried material was extracted. If less than 10 g of material was available, the entire sample was extracted minus a small portion retained as a reference. The tissue extracted varied from sample to sample. In many cases, because of small sample size and

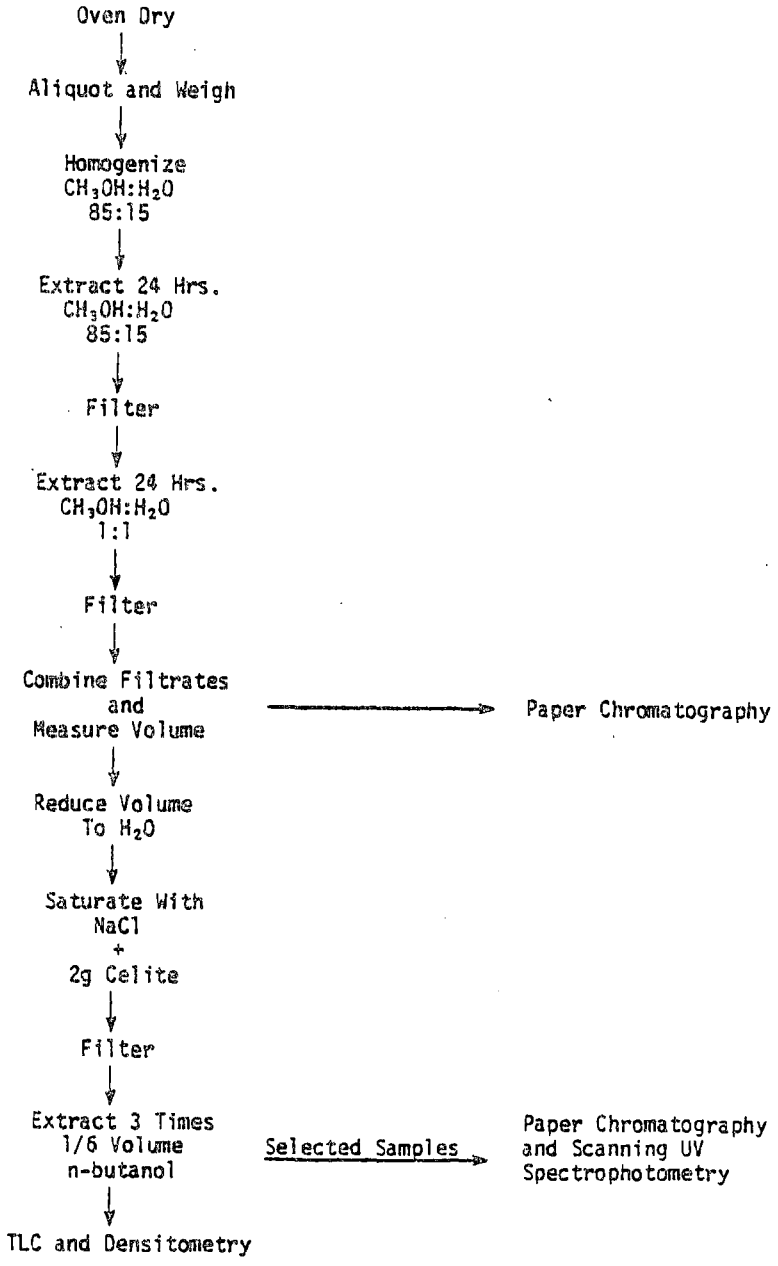


Figure 4-1. Analytical Flow Diagram for the Analysis of Flavonoids in Quail Food Plants

variations in maturity, analogous tissues from each sample could not be extracted. The extraction procedure used was:

1. Determine dry weight of sample to be extracted.
2. Homogenize sample for 15 minutes with 10 mL/g $CH_3OH:H_2O$ 85:15. A Virtis blade-type tissue homogenizer was used for homogenization.
3. Quantitatively transfer homogenate to 250 mL Erlenmyer flask and place on orbital shaker for 24 hours. Note if soil residue remained in homogenizer flask, dry, weigh and subtract from sample weight.
4. Filter sample and reextract for 24 hours with $CH_3OH:H_2O$ 1:1 with shaking.
5. Filter sample and combine with filtrate from Step 4.
6. Determine final crude extract volume and reserve volumetric aliquot for paper chromatography.
7. Reduce sample volume to approximately 50 mL by evaporation at 80° C.
8. Saturate the extract with NaCl and add 2-3 g celite for chlorophyll removal.
9. Filter aqueous extract.
10. Partition aqueous extract serially with 3 X 1/6 aqueous extract volume n-butanol.

11. Determine volume of n-butanol extract and transfer to 50 mL crimp-seal hypovial with Teflon-faced septum and refrigerate until analysis.

In addition to the above procedure, aliquots of some samples were taken to dryness at 80° C under a stream of dry nitrogen and redissolved in one-fifth volume of either pyridine or dimethylsulfoxide (DMSO) to concentrate the samples because precipitation occurred when concentration of the n-butanol extract was attempted. While this was successful as a concentration step, the procedure was not used because of solvent interference with the thin-layer chromatography (TLC) as described in Section 4.3. Therefore, the dilute n-butanol extracts were used for TLC and densitometric analysis.

4.2 Paper Chromatography

For initial extract characterization, aliquots of the crude CH_3OH:H_2O extracts were subjected to paper chromatography using the standard procedure of Mabry, et al. (Ref. 8). The sample was spotted on the lower right hand corner of a 46X57 cm Whatman 3 MM chromatographic paper using a Pasteur pipette until the spot appeared deep purple when viewed with ultraviolet (UV) light at a wavelength of 370 nm. Descending development of the chromatogram was accomplished using a standard chromatography cabinet as described in Reference 8.

The chromatogram was initially developed for approximately 24 hours with tertiary butanol:glacial acetic acid:water (TBA) in a ratio of 3:1:1. After drying, the chromatogram was developed in the second dimension for approximately eight hours with glacial acetic acid:water (HOAc) in a ratio of 15:85. After drying, the chromatogram was viewed with UV light at 370 nm, then fumed with ammonia and viewed to assess sample complexity and gain preliminary qualitative data.

In addition, the n-butanol extract from a representative Astragalus nuttallianus (Andrews site, 4-22-81) was chromatographed on six chromatographic papers using the described procedure. Individual spots were cut out and extracted for 24 hours with methanol. The methanol extracts were filtered, concentrated by vacuum evaporation and reserved for scanning UV analysis.

4.3 Thin-Layer Chromatography

The n-butanol extracts from all plant samples were chromatographed by two-dimensional thin-layer chromatography (TLC) after the techniques of Wilkins and Bohm (Ref. 10). TLC plates were made by first cleaning a 20X20 cm glass plate with dichromate/sulfuric acid solution and deionized water and then coating the plate with a 0.25 mm layer of MN Polyamid DC 6.6. Eleven grams of polyamide were slurried with 62 mL deionized water and 14 mL methonol provided sufficient material for preparation of five plates.

Quantitative spotting in the lower right hand corner of the plate was accomplished using a 5μL Drummond microdispenser. The TLC plate was placed on a hot plate at 70° C to facilitate spotting. Repeated applications of the extract totaling from 1-40 μL were spotted depending on the flavonoid concentration of the extract. In most cases at least two plates per sample were spotted to assure development of at least one plate suitable for analysis.

The TLC plates were developed ascendingly in two dimensions using standard glass TLC tanks fitted with glass lids and two 20X20 cm saturation pads. The plates were initially developed for approximately six hours with distilled water:n-butanol:acetone:1,4-dioxane in a ratio of 210:45:35:20 (polar dimension). After oven-drying overnight at 50° C, the plates were developed in the second dimension with benzene:methanol:2-butanone:distilled water in a ratio of 55:20:22:3 (nonpolar dimension). All organic solvents were spectral quality.

After development, the flavonoids were visualized by spraying the TLC plates with a solution of 0.1 g diphenylboric acid, ethanolamine complex (Natural Products Reagent A) in 100 mL of spectral grade methanol. The flavonoids appeared as yellow, yellow-orange, yellow-green or green spots.

In addition, test plates were developed using DMSO or pyridine as the spotting solvent. Both solvents unacceptably interfered with the separation of the compounds. This was true of pyridine even when the spot was treated with 0.2 N HCl in an attempt to reprotonate the flavonoids. Higher concentrations of HCl caused hydrolysis of the flavonoids.

Finally, samples of Kochia scoparium and Lepidium virginicum contained only highly glycosylated flavonoids. These compounds were insufficiently mobile in the nonpolar phase to allow resolution from the baseline streak which interfered with analysis. In an attempt to separate these compounds from interfering compounds, these extracts were also spotted on 20X20 cm Whatmann 0.25 mm K2 cellulose plates and developed with distilled water:glacial acetic acid in a ratio of 95:5. This one-dimensional technique was also not successful in resolving the flavonoids from other interfering compounds. Because of this poor resolution, these samples could not be measured densitometrically.

4.4 Densitometry and Quantitation

All quantitative determinations were made on the developed thin-layer plates using a Farrand UV-VIS-2 densitometer. A series of six compounds, isorhamnetin 3-galactoside, luteolin, vitexin, 7,4'-dimethyl kaempferol, rutin and genestein, were spotted and sprayed with Natural Products Reagent A. The absorbance spectra of these compounds were determined in-situ to ascertain the optimum wavelength for measuring sample compounds. A wavelength of 420 nm was chosen as most representative. All sample spots except those which turned dull orange with Natural Products Reagent A showed good response at this wavelength. Compounds which turned dull orange were relatively rare in sample chromatograms and were never major constituents. Therefore, the poor instrument response to these compounds

did not significantly affect the quantitative results. The instrument parameters for quantitative measurement were:

Mode: Absorbance, single beam

Lens Aperture: 0.625 in

Sample channel aperture: 0.021 in

Sample channel slit width: 11/32 in

Slit bandwidth: 10 nm

Excitation beam attenuation: 7

Exciter monochromater wavelength: 420 nm

Spatial scan speed: 2 in/min

Gain: 150

The integrated areas of the TLC spots were determined using a Hewlett Packard Model 3390A plotting integrator, typically in valley-to-valley baseline mode.

TLC spots were quantitated relative to rutin. Rutin was spotted and developed at concentrations of 0.0, 0.38, 0.95 and 2.87 µg in the same manner as the sample chromatograms. The rutin plates were analyzed daily for calibration of the instrument. A daily calibration curve was calculated using a correlation coefficient of 0.99 as the acceptance criterion. A typical rutin calibration curve is shown in Figure 4-2.

Each sample spot was measured twice. A mean and a standard deviation for each spot were calculated after quantitation of the two measurements using the linear regression curve for rutin.

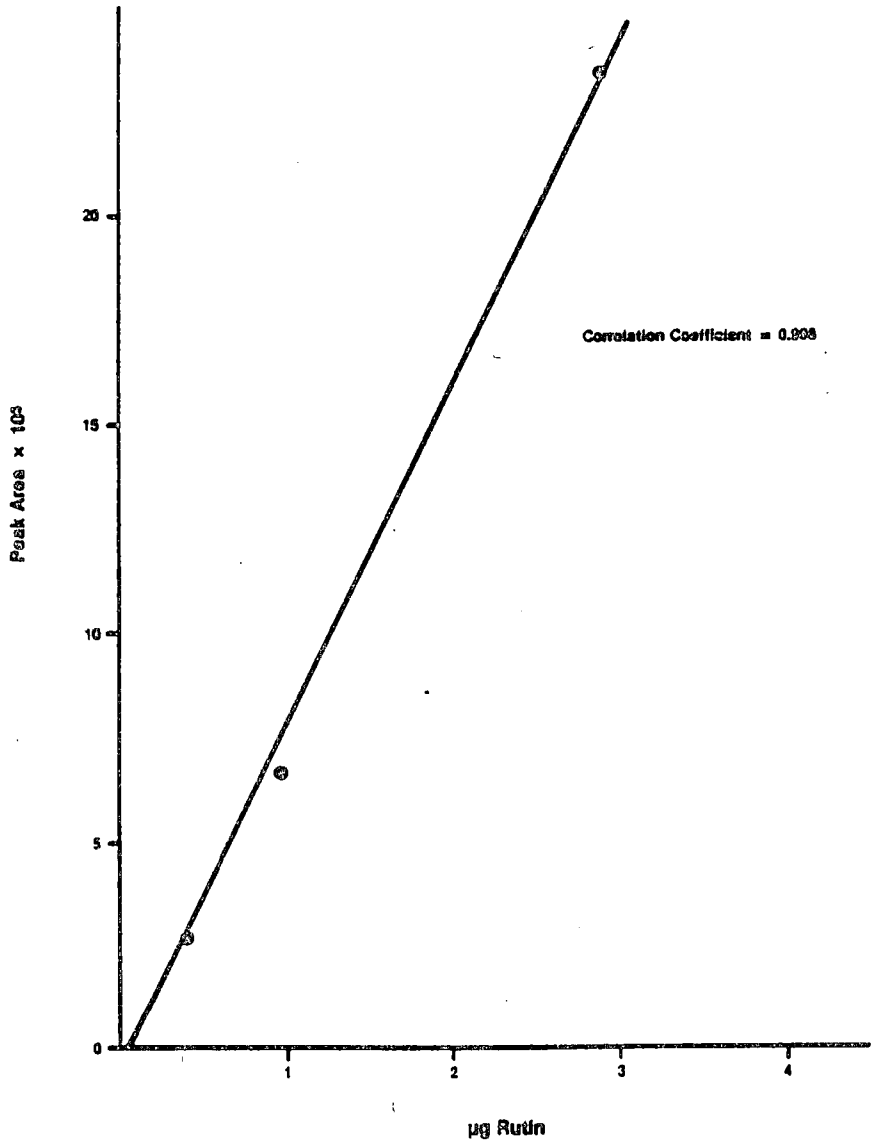


Figure 4-2. Linear Regression for Rutin Quantitated by In-Situ Absorbance Densitometry

The equations used for quantitation were:

$$1.\quad A = \frac{B}{C} \times 1000 \times \frac{D}{E}$$

Where: A = µg/g flavonoid in the dried plant tissue

B = µg compound on the plate from rutin linear regression

C = µL n-butanol spotted

D = mL n-butanol extract

E = grams plant tissue represented in n-butanol extract

and

$$2.\quad \bar{X}_T = \frac{\sum \bar{X}_A}{n} \pm \sqrt{\sum \sigma_A^2}$$

Where : $\bar{X}_T$ = total µg/g flavonoid in the plant tissue relative to rutin

$\bar{X}_A$ = mean µg/g plant tissue of individual spots from Equation 1

σ_A = standard deviation of individual spots (The more conservative estimate was used rather than $\frac{\sigma_A^2}{n}$)

n = number of spots

Similarly, average µg/g plant tissue values for each collection date at each site were calculated using:

$$3.\quad \bar{X}_S = \frac{\sum \bar{X}_T}{n} \pm \sqrt{\frac{\sum V^2}{n}}$$

Where: $\bar{X}_S$ = average µg/g flavonoid in all samples for each individual collection date at each site

$\bar{X}_T$ = mean µg/g flavonoid in individual samples from Equation 2

V = individual sample error from Equation 2

n = number of individual samples

Weighted means were calculated for each site using:

$$4.\quad \bar{X}_W = \frac{\sum \bar{X}_S (n)}{n} \pm \sqrt{\frac{\sum (V(n))^2}{\sum n^2}}$$

Where: $\bar{X}_W$ = the weighted mean for total flavonoid concentrations at each site

$\bar{X}_S$ = mean flavonoid concentration for each collection date from Equation 3

n = number of samples contributing to $\bar{X}_S$ for each collection date

V = total analytical variance for each collection date from Equation 3

Linear regressions and weighted linear regressions were calculated using standard statistical methods. Outliers were tested by applying the F test to the valued derived from:

$$5. \quad K = \frac{\sigma_R^2}{\sigma_S^2}$$

Where: K = ratio to be tested by F test

σ_R = standard deviation of residuals = $\sqrt{\frac{\sum (Y - Y')^2}{(n - 1)}}$

Where: Y = experimental flavonoid concentration for a given X (X = date or soil moisture value)

Y' = predicted flavonoid concentration for each X on the regression line of X_1, Y_1, ... X_n, Y_n

n = total number of experimental determinations contributing to the regression line

σ_S = σ_R minus the suspected outlier

4.5 Scanning Ultraviolet Spectrophotometry

Scanning ultraviolet (UV) spectra of spots from Astragalus nuttallianus (See Section 4.2) were determined using the standard procedure of Mabry, et al. (Ref. 8). Spectra were determined in methanol. The series of spectra that were determined for each spot were:

1. Methanol only
2. Methanol + sodium methoxide
3. Methanol + sodium methoxide after 5 minutes
4. Methanol + aluminum chloride
5. Methanol + aluminum chloride + hydrochloric acid
6. Methanol + sodium acetate.

In addition the purity of the rutin standard was determined by measurement of a standard solution of commercially prepared rutin in ethanol at 359 nm.

All UV spectra were measured using a Varian Cary 219 double beam spectrophotometer.

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